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Characterisation of Fractalkine/CX3CL1 and Fractalkine Receptor (CX3CR1) Expression in Abdominal Aortic Aneurysm Disease

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Abstract *Objectives:* Fractalkine (CX3CL1) promotes adhesion and extravasation of leucocytes through interactions with fractalkine receptor (CX3CR1) expressed on $CD56^+/CD16^+$ NK cells and $CD8^+$ T cells. The current study aims to test the hypothesis the CX3CL1–CX3CR1 interaction contributes to the inflammatory infiltrate in AAA tissue.
Design and methods: Immunohistochemistry (IHC) was used to define expression of CX3CR1 in AAA tissue. Multi-parametric flow cytometry (FC) was used to determine CX3CR1 expression on T-cells ($CD3^+$) and NK cells ($CD56^+$) from AAA tissue and peripheral blood of AAA patients and healthy controls. Regulation of CX3CL1 expression by vascular endothelial (vEC) and smooth muscle cells (vSMC) was examined in vitro using primary cell cultures.
Results: $CX3CR1^+$ cells were detected in 19/28 AAA tissue samples and predominately localised in the adventitia. PBMCs from patients with AAA demonstrated higher percentages of $CX3CR1^+$ NK cells (60.0–88.6%) and T cells (7.5–39.4%) compared with healthy controls. Furthermore, the frequency of $CX3CR1^+$ NK cells (91%) and T cells (94%) in inflammatory AAA tissue were higher than in atherosclerotic AAA tissue. The pro-inflammatory cytokine TNFα increased expression of fractalkine by vSMC and vEC.
Conclusion: $CX3CL1^+$ and $CX3CR1^+$ cells are present in AAA disease and their interaction may contribute to the recruitment of inflammatory cells seen in AAA tissue.

Introduction

Abdominal aortic aneurysm (AAA) is a complex, multifactorial vascular disorder characterised by a localised dilation of the aorta. The pathogenesis of aneurysm formation is

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poorly understood, however, the progressive loss of elastic tissue and well-organised smooth muscle cell layers may be attributed to an inflammatory process.[1,2] Our group has previously demonstrated that the majority of haematopoietic cells within the cellular infiltrate are T cells (58.1 $\pm$ 5.3%) and B cells (41.1 $\pm$ 5.7%), with NK cells and macrophages present in low amounts (7.3 $\pm$ 2.5% and 2.1 $\pm$ 1.4% respectively).[3] The process by which these cells migrate into the AAA tissue remains unclear.

Chemokines, chemoattractant cytokines synthesised at sites of inflammation, regulate leukocyte trafficking through interactions with G protein-coupled receptors.[4] Recent evidence suggests that the chemokine fractalkine (CX3CL1) plays an important role in inflammatory vascular disorders.[5,6] Fractalkine is a unique chemokine with an extracellular domain of 76 amino acids including a novel CX3C conserved cysteine motif.[7]

Vascular endothelial cells (vECs) express membrane-bound fractalkine in response to proinflammatory stimuli including TNFα (tumour necrosis factor-α) and IFNγ (interferon-γ).[8] IFNγ, recently described in the development and propagation of AAA,[9] has also been shown to induce expression of fractalkine on cultured aortic smooth muscle cells *in vitro*.[10]

Cell surface fractalkine promotes capture of leucocytes through interactions with CX3CR1 expressed on CD16$^+$ NK cells, CD8$^+$ T cells and CD14$^+$ monocytes.[11] Additionally, fractalkine increases NK cell adhesion and cytotoxicity towards endothelial cells.[12] The resulting injury to the endothelial barrier may further amplify the migration of leucocytes.

Previous animal[13] and human studies[5,6] have demonstrated a reduction of atherosclerosis in mice following targeted deletion of CX3CR1 and increased expression of CX3CL1 and CX3CR1 in patients with coronary artery disease (CAD). However, the expression of fractalkine and CX3CR1 in patients with AAA has not previously been investigated. Therefore the aim of the present study was to characterise the expression of CX3CR1 by AAA tissue and peripheral blood mononuclear cells (PBMCs) of patients with AAA.

Materials and Methods

Patients and samples

Forty three patients admitted for elective infra-renal AAA repair were studied (Table 1). From these patients, samples of AAA tissue (eleven) and peripheral blood (seventeen) were taken specifically for this study and a further 17 archival AAA tissue samples were available for inclusion. Patients with atherosclerotic AAA and two patients with inflammatory AAA identified by highly elevated C-reactive protein, grossly thickened AAA wall and appearance at surgery were included. Patients with diabetes, those receiving immunosuppressive drugs and patients with previous or concurrent malignant disease were excluded. Nine age-matched control patients admitted to hospital for minor surgical procedures were also studied. Control patients had no history of cardiovascular disease or peripheral vascular disease and were not on statin medication. To date there have been no reports of sex-related differences in fractalkine and CX3CR1 expression. Local Research Ethics Committee approval was obtained and all individuals gave their informed consent.

Table 1 Patient demographics

Criteria	AAA patients	Control patients
Number	43*	9
Median age, years (IQR)	74 (70–78)	67 (63–71)
Men, *n* (%)	40 (93)	6 (67)
Median AAA diameter, cm (IQR)	6.1 (5.6–7.2)	n/a
Median lymphocyte number $\times 10^6$/ml (IQR)	4.1 (3.4–5.6)	5.0 (4.3–6.8)
Current or ex-smoker, *n* (%)	38 (88)	2 (22)
Hypertension, *n* (%)	29 (67)	1 (11)

Abbreviations: AAA, abdominal aortic aneurysm; IQR, interquartile range.
* 2 patients with inflammatory AAA.

Cell isolation

Tissue mononuclear cells (MNCs) were isolated from fresh AAA sections as previously described.[3] Briefly, biopsies were cleaned by agitation in phosphate-buffered saline (PBS), diced using a 35 μm tissue homogeniser (Medimachine, Dako, UK) and filtered through a 70 μm filter (Becton Dickinson, UK). Viable PBMCs and tissue MNCs were isolated by density gradient centrifugation using Ficol-Isopaque (Axis-Shield, Norway). The viability of isolated cells, assessed by trypan blue dye exclusion, was routinely >90%.

Stromal cell culture

Human vECs, vSMCs (saphenous vein) and aSMCs (AAA) were cultured by a modification of previously described methods.[14,15] Briefly, segments of saphenous vein were obtained from patients undergoing bypass surgery with no evidence of AAA and used as a source of vECs and vSMCs. vECs were isolated and incubated at 37 °C with collegenase (Worthington, Lorne Laboratories, UK) for 10 min followed by washing in DMEM supplemented with 5% fetal calf serum (FCS) (LabTech International, UK). The cell pellet was resuspended in complete endothelial culture medium; M199 (Sigma Aldrich, Dorset) supplemented with 20% FCS, 100 μg/mL penicillin, 100 μg/mL streptomycin, 15 μg/mL ECGS, 1 mM pyruvate (Sigma Aldrich, Dorset), 2 mM L-Glutamine, 20 mM HEPES (Invitrogen, UK) and 5 units/mL heparin (Leo Laboratory, UK) and grown to confluence. SMCs were prepared using explant technique. Vessel segments were maintained in DMEM medium supplemented with 10% FCS, 100 μg/mL penicillin, 100 μg/mL streptomycin, 2 mM L-Glutamine at 37 °C in a humidified incubator in 5% CO_2 in air. After 4–5 weeks cells were passaged and grown to confluence. All cells were used between passages 2 and 5.

Immunohistochemistry

3 $\times$ 1 cm section of fresh AAA tissue was sampled from the anterior aortic wall immediately after clamping the neck

of the aneurysm and the common iliac vessels. Samples were used for immunohistochemical analysis ($n = 31$) and isolation of MNCs ($n = 2$). Formalin-fixed AAA tissue was embedded in paraffin and sectioned (5 μm). A series of experiments using tonsil tissue were performed to determine optimal antibody concentrations and incubation times for the immunohistochemical detection of CX3CR1.[16] Briefly, dewaxed and rehydrated sections were incubated with 0.3% H_2O_2 for 10 min at 25 °C to block endogenous peroxidase activity prior to antigen unmasking by microwaving (950 watts power) in 0.01 M sodium citrate buffer (pH 6.0) for 5 min followed by rapidly cooling on ice for 3 min. Endogenous biotin was blocked using avidin biotin blocking kit (Vector Laboratories, UK). All antibodies were diluted using antibody buffer TNB (0.1 M Tris, 0.15 M NaCl pH 7.5). After blocking non−specific binding with rabbit IgG (1:100, Vector Laboratories, UK) sections were incubated for 1 h at 25 °C with a rabbit anti-CX3CR1 antiserum (1:100, Abcam, UK) or rabbit serum (1:100, Dako, UK) used as a negative control. Biotinylated anti-rabbit IgG (1:100, Dako, UK) was applied for 30 min, followed by horseradish peroxidase conjugated avidin-biotin complexes (Vector Laboratories, UK). Immunodetection was performed using the peroxidase substrate 3,3′-diaminobenzidine (Vector Laboratories, UK). Tissue sections were counterstained with Harris haematoxylin, dehydrated through ethanol and xylene, mounted and examined with a Zeiss microscope (Carl Zeiss, UK). Images were captured using AxioVision software (Carl Zeiss, UK). Five fields of view from the media and advential areas were assessed for positive CX3CR1 staining using a three-plus system[16] with the presence of CX3CR1 staining in 4 or 5 fields of view reported as high (+++), 2 or 3 fields of view reported as medium (++) and 1 field of view reported as low (+).

Antibody staining and flow cytometry

PBMCs and tissue MNCs were incubated for 1 h at 25 °C with saturating amounts of commercial monoclonal antibodies conjugated to biotin or a fluorochrome: anti-CD45 (BD Biosciences, UK), anti-CD3 (clone S4·1) (Caltag, USA), anti-CD56 (clone C5.9) (Dako, UK), anti-CD34 (Caltag, USA), anti-myosin (Sigma-Aldrich, Germany) and anti-CX3CR1 (Abcam, UK). Cells were washed and incubated for 30 min at 25 °C with: FITC-conjugated swine anti-goat IgG (Caltag, USA), biotin-conjugated anti-mouse IgG (Dako, UK) or biotin-conjugated anti-rabbit IgG (Dako, UK). Bound biotinlyated antibodies were visualised by incubating with Streptavidin-FITC (1:100, Caltag, USA), Streptavidin-PE (1:100, BD Biosciences, UK) or Streptavidin-AF 633 (1:100, Molecular Probes, UK) for 30 min at 25 °C. Cells were washed and analysed on a FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson, UK) analysing 5000 electronic events for each sample. Electronic gating of cell populations was used to exclude tissue and cell debris.

Human recombinant TNFα was added to cultured vSMCs (10 ng/ml) and vECs (100 ng/ml) for 12−16 h.[10,17] After trypsin detachment, non-stimulated and stimulated cells were washed in PBS and incubated with goat anti-human fractalkine antibody (1:25, R&D Systems, UK) or goat Ig (1:25, Dako, UK) for 1 h. After washing cells were incubated for 30 min with FITC-conjugated swine anti-goat IgG (1:50, Caltag, USA). vECs were also stained with antibodies to human CD34 (1:50 Caltag, USA),[18] for 1 h followed by incubation with Streptavidin-PE (1:100, BD Biosciences, UK) for 30 min. Cells were then either analysed using flow cytometry (FC) or were fixed for 12−16 h in 100 μl 0.1% paraformaldehyde at 4 °C for intracellular staining with anti-myosin heavy chain antibody (1:10, Sigma-Aldrich, Germany).[19] For the detection of human myosin heavy chain cells were incubated with biotin conjugated anti-mouse IgG (1:25, Dako UK) followed by Streptavidin-FITC (1:100, Caltag, USA) and analysed using FC as described above.

Statistical analyses

All values are given as percentage median values with interquartile ranges. The Mann-Whitney Test was performed with SPSS for Windows 12.0 software with $P < 0.05$ considered statistically significant.

Results

Localisation of CX3CR1$^+$ cells in AAA tissue

AAA tissue samples collected for this study (eleven) and archival AAA tissue samples (seventeen) were assessed by immunohistochemistry. An inflammatory infiltrate was present in the media and adventitia of all AAA's. Fractalkine receptor positive (CX3CR1$^+$) cells were detected in 19 out of 28 AAA's with the highest amount of staining demonstrated in the adventitia (Fig. 1E). CX3CR1$^+$ cells were detected in the adventitia of sixteen samples, of which four contained high numbers of CX3CR1$^+$ cells (stained cells present in 4 or 5 fields of view) and five contained fewer CX3CR1$^+$ cells (stained cells present in 2 or 3 fields of view). Seven samples contained CX3CR1$^+$ cells in the media (Fig. 1F), with one sample containing positive cells in ≥2 fields of view. No staining was seen in AAA tissue stained with control antibodies (Fig. 1A−D).

Distribution of CX3CR1$^+$ cells in the blood of AAA patients and healthy controls

To quantify and determine the phenotype of cells expressing CX3CR1 flow cytometry of PBMCs was carried out using anti-CX3CR1 antibodies in conjunction with antibodies that identify haematopoetic cells (CD45), T cells (CD3) and NK cells (CD56) among PBMCs from AAA patients ($n = 17$) and healthy controls ($n = 9$). Availability of reagents determined the number of samples stained. As previously reported[3] AAA patients had elevated numbers of peripheral blood NK cells; 20.7% (13.9−27.2%) in AAA patients compared to 15.7% (14.2−27.4%) in healthy controls.

A representative staining profile of PBMCs from an individual AAA patient is shown in Fig. 2 with the distribution of CX3CR1$^+$ cells in PBMCs of all AAA patients and controls shown in Fig. 3. The frequency of CX3CR1$^+$ haematopoietic cells was similar in patients with AAA (26.2, 23.7−28.7%) and healthy controls (25.5, 19.3−27.8%), however AAA patients demonstrated increased CX3CR1 expression among NK cells and T cells. Patients with AAA had 77.1%

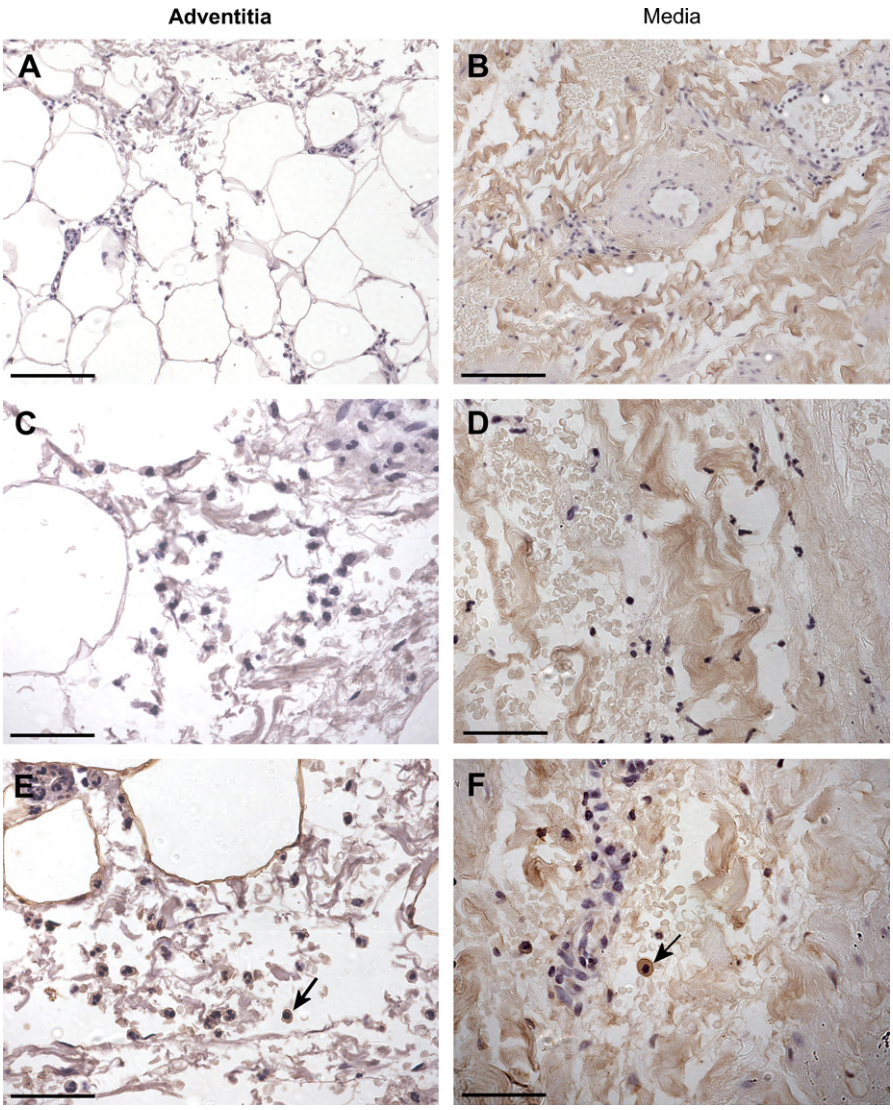

Figure 1 Localisation of fractalkine receptor (CX3CR1) in AAA. Adventitia and media stained with a control antibody (A—D) and with anti-CX3CR1 antibody (E and F) with the arrows indicating representative $CX3CR1^+$ cells. Images taken from 2 samples of AAA tissue from a total of 28 AAA tissue samples analysed. Scale bar = 40 μm, Magnification ×16 (A and B), ×40 (C—D).

(60.0—88.6%) of $CD56^+$ NK cells expressing CX3CR1 compared to 56.7% (50.4—73.1%) by $CD56^+$ NK cells from control patients. Furthermore, the percentage of $CD3^+$ T cells expressing CX3CR1 was 5.8% (1.7—13.6%) in healthy controls and 13.2% (7.5—39.4%) in patients with AAA (Fig. 3). Although the differences were not significant ($p > 0.05$), there was an overall increased expression of CX3CR1 in patients with AAA in comparison to healthy controls.

$CX3CR1^+$ cells in AAA tissue

Using flow cytometry we compared the distribution of CX3CR1 expression among tissue mononuclear cells (MNCs) isolated from single samples of atherosclerotic and inflammatory AAA tissue (Fig. 4). Virtually all (96%) $CD45^+$ haematopoietic MNCs from the inflammatory AAA tissue sample expressed CX3CR1, whereas only 12% of the atherosclerotic AAA MNCs were $CX3CR1^+$ (Fig. 4). In comparing expression of CX3CR1 by $CD3^+$ T cells and $CD56^+$ NK cells in the inflammatory AAA and atherosclerotic AAA infiltrate it was clear that whereas the majority of T cells and NK cells from inflammatory AAA expressed CX3CR1 only a minority of these cells in atherosclerotic AAA were $CX3CR1^+$ (Fig. 4).

Regulation of fractalkine expression by vSMCs and vECs

To further investigate expression of membrane-bound fractalkine on SMCs and ECs, primary cultures of vSMCs, vECs and aSMCs established from tissue explant cultures were examined. TNFα, a pro-inflammatory stimulus demonstrated in AAA tissue[20] was used to stimulate expression of fractalkine as previously described.[10,17]

FC analysis revealed increased expression of fractalkine among all 3 cell types following exposure to TNFα (Fig. 5).

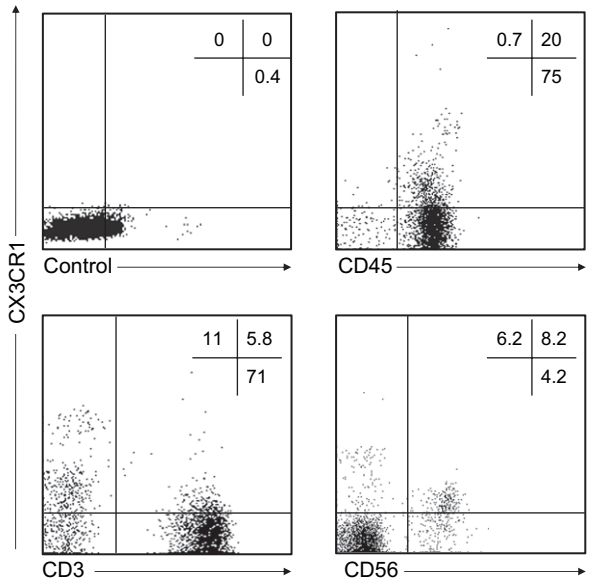

Figure 2 Phenotype of CX3CR1$^+$ PBMCs in atherosclerotic AAA. PBMCs were stained with antibodies to CX3CR1, CD45, CD3 and CD56, and analysed by flow cytometry. The four quadrants were set using isotype matched control antibodies and the values shown represent the percentage of leucocytes in each quadrant. Cells expressing CX3CR1 are shown in the upper right hand quadrant with those lacking expression of CX3CR1 shown in the bottom right hand quadrant. The results shown are representative of those obtained from 18 AAA patients and 9 control subjects.

The greatest increase in CX3CR1 expression was seen in vECs, increasing from 4.5% CX3CR1$^+$ cells to 66.7% CX3CR1$^+$ following stimulation with TNFα. vSMCs and aSMCs increased CX3CR1$^+$ cells by 1.5 and 1.2 fold respectively following stimulation with TNFα.

Discussion

This study is the first to investigate expression of fractalkine and CX3CR1 in AAA. We have identified CX3CR1$^+$ cells in the cellular infiltrate of AAA tissue as being predominately T cells and NK cells, with greater expression seen in inflammatory AAA in comparison to atherosclerotic AAA. Furthermore, primary vSMCs and vECs could be induced to express fractalkine. Although this study is limited by the sample size, our findings suggest expression of fractalkine may be induced by inflammatory stimuli and therefore may contribute to the pathogenesis of AAA by mediating extravasation of leucocytes.

CX3CR1$^+$ cells were present in both the media and adventitia of AAA tissue to varying degrees. This may be due to limitations of immunohistochemistry and specimens taken at different stages of the disease process, however, the results are consistent with CX3CR1 involvement in leucocyte recruitment to aortic tissue. Traditionally, extravasation of leucocytes has been described via the luminal aspect of the vessel, however, recent studies have proposed neovascularisation in aneurysmal tissue may permit infiltration of leucocytes in AAA from the adventitia.[2,21] Our findings of substantial infiltrate with evidence of angiogenesis in both structural areas of AAA tissue supports this theory.

Flow cytometry demonstrated considerable difference in CX3CR1$^+$ cells in inflammatory and atherosclerotic AAA tissue, although the data was based on single samples. Histologically, an inflammatory AAA is considered to be at one extreme of a progressive continuum of increasing inflammation involved in the pathogenesis of all AAAs.[22] This extreme may be reflected in the high levels of CX3CR1$^+$ cells in the inflammatory AAA.

Animal and human studies favour the concept of a T-cell mediated and IFNγ triggered pathophysiological process in AAA.[9,23] Interaction between CD16$^+$ peripheral blood monocytes and CX3CL1$^+$ endothelial cells results in

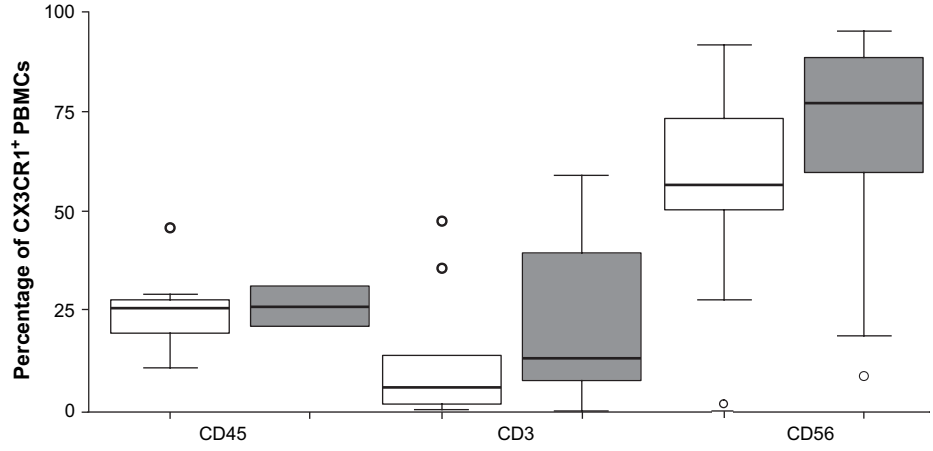

Figure 3 Distribution of CX3CR1$^+$ PBMCs in patients with atherosclerotic AAA and healthy controls. White boxes represent control subjects ($n = 7, 9, 9$ for CD45, CD3 and CD56 respectively). Grey boxes represent AAA patient samples ($n = 2, 16, 17$ for CD45, CD3 and CD56 respectively). Box plot components are: horizontal line, median; box, interquartile range; whiskers, 5 and 95 percentiles; circles, outliers.

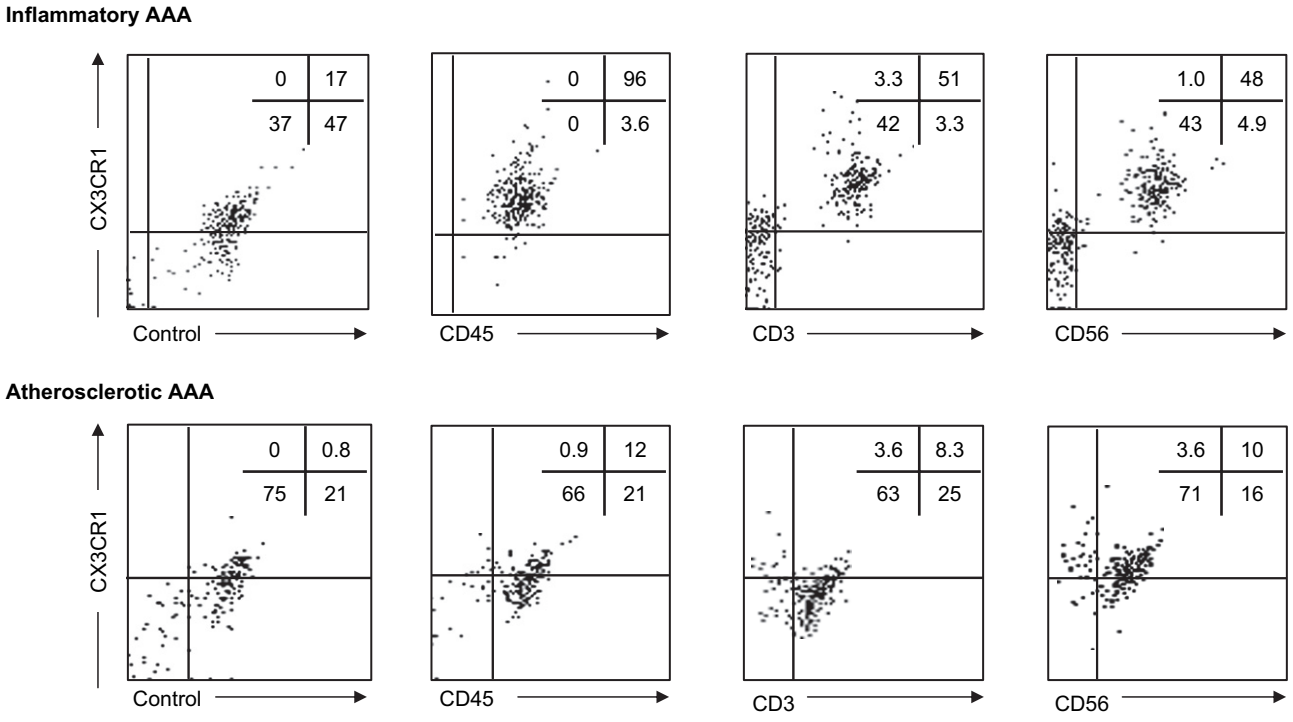

Figure 4 Phenotype of CX3CR1$^+$ cells in atherosclerotic and inflammatory AAA. Tissue mononuclear cells were stained with antibodies to CX3CR1, CD45, CD3 and CD56 and analysed by flow cytometry. Quadrants were set using isotype matched control antibodies with numbers representing percentage cells positive in each quadrant. Cells expressing CX3CR1 are shown in the upper right hand quadrant with those lacking expression of CX3CR1 shown in the bottom right hand quadrant. The results are from single atherosclerotic and inflammatory AAA tissue samples.

increased production of cytokines and the matrix metalloprotease 9, a molecule associated with aneurysmal expansion and rupture.[24] Cells exhibiting other features of cytolytic activity including intracellular perforin and granzme B, have also been reported to show chemotaxis towards soluble fractalkine and high expression of CX3CR1.[25] Additionally dendritic cells, described at the adventitial level of the inflammatory infiltrate in AAA express CX3CR1 mRNA and show chemotaxis towards fractalkine.[26,27] This suggests fractalkine may contribute to the pro-inflammatory state and the recruitment of leucocytes reported in AAA tissue.

Among PBMCs, AAA patients demonstrated increased CX3CR1$^+$ cells in comparison to control subjects, although the difference was not significant ($p > 0.05$). Potential confounding factors include smoking and hypertension, both of which were significantly increased in AAA patients. These risk factors for AAA can contribute to a pro-inflammatory state[28,29] potentially including an increased expression of CX3CR1, which maybe further modified by anti-hypertensive medication in the AAA patient group.[5]

CX3CR1 expression was most notable on CD56$^+$ NK cells in the peripheral blood and tissue of AAA patients. Increased cytotoxicity and circulating levels of NK cells have previously been described in AAA patients suggesting NK cells have an important role in AAA disease.[3,6] Yoneda *et al*. reported fractalkine increased NK cell adhesion and vEC susceptibility to NK cell mediated cytolysis.[12] Our group has previously shown that the NK cells present in AAA do not secrete IFNγ but have significant cytoxicity directed at vSMCs therefore fractalkine may affect the cytotoxity of NK cells by increasing release of the lytic mediators perforin and granzymes.[3]

The difference in CX3CR1 expressing cells in peripheral blood and AAA tissue implies the role of the receptor may vary between tissues. Studies of CX3CR1 expression in other vascular inflammatory disorders have reported the majority of CX3CR1$^+$ cells were CD3$^+$CD8$^+$ T cells amongst PBMCs in patients with CAD[5] and SMCs in an atherosclerotic plaque[6] suggesting the role of fractalkine and CX3CR1 expressing cells maybe specific to the inflammatory disease and tissue.

Results from this ex-vivo study identify TNFα, a pro-inflammatory stimuli demonstrated in AAA tissue,[20] as a stimulator of fractalkine expression on cultured ECs and SMCs as previously shown.[8,30] Stimulated aSMCs also demonstrated fractalkine expression, however, the increase in expression was much lower than that seen in non-diseased vSMC. This may be due to maximum stimulation of cells from AAA tissue *in situ* and as a result cells may be refractory to further inductive stimuli. Alternatively, aSMCs may require the presence of other synergistic signals such as pro-inflammatory cytokines for the expression of fractalkine.[31] It is also important to note these results are based on cells obtained from only a single AAA tissue sample consequently there may be variability of fractalkine expression as observed in previous experiments.[16]

The presence of fractalkine on vECs and CX3CR1$^+$ cells in both AAA tissue and PBMCs suggests fractalkine contributes to the development of AAA disease. However, it is unclear whether cells expressing fractalkine and CX3CR1 drive the

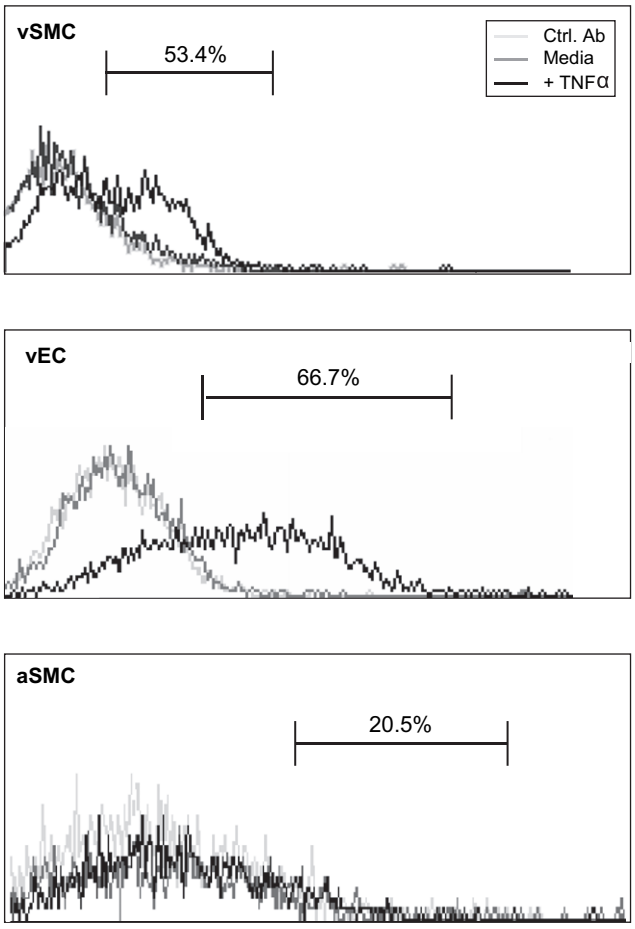

Figure 5 Upregulation of fractalkine by vascular stromal cells in response to TNFα. Primary cultures of vascular and aortic smooth muscle cells (vSMC, aSMC) were incubated with 10 ng/ml TNFα for 12—16 h and vascular endothelial cells (vEC) with 500 ng/ml TNFα for 12—16 h. The values shown represent the proportion of cells expressing fractalkine following stimulation.

formation of AAA or if initiation of AAA promotes expression of fractalkine and CX3CR1. Current theories suggest an antigenic stimulus e.g. damage to the vECs or presence of elastin-derived peptides triggers the production of cytokines in the aortic wall.[32] vSMCs and ECs are activated by this or an alternative stimuli e.g. infection with *Chlamydia pneumonia*[33] and increase production of membrane-bound fractalkine promoting the extravasation and retention of $CX3CR1^+$ leucocytes into the aortic tissue. The high proportion of $CX3CR1^+$ NK cells suggests fractalkine-mediated recruitment into AAA tissue contributes to the large population of NK cells demonstrated in the cellular infiltrate of AAA.[3] Within the aneurysm tissue, fractalkine may promote increased NK cell cytotoxicity causing damage to the structure of the aorta and therefore potentiating aneurysm formation. Questions raised by this model centre around the contribution fractalkine makes to the inflammatory process of AAA. It is not known whether or not there is a specific stimulus for vSMCs and vECs in AAA triggering fractalkine expression or if fractalkine is the initiating mediator for leucocyte recruitment. Importantly, the affect of fractalkine on the function of inflammatory cells and in particular NK cell cytoxicity in AAA disease remains unclear.

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